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Use of Biochemical Kinetic Data To Determine Strain Relatedness among *Salmonella enterica* subsp. *enterica* Isolates

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Classical biotyping characterizes strains by creating biotype profiles that consider only positive and negative results for a predefined set of biochemical tests. This method allows *Salmonella* subspecies to be distinguished but does not allow serotypes and phage types to be distinguished. The objective of this study was to determine the relatedness of isolates belonging to distinct *Salmonella enterica* subsp. *enterica* serotypes by using a refined biotyping process that considers the kinetics at which biochemical reactions take place. Using a Vitek GNI+ card for the identification of gram-negative organisms, we determined the biochemical kinetic reactions (28 biochemical tests) of 135 *Salmonella enterica* subsp. *enterica* strains of pig origin collected in Spain from 1997 to 2002 (59 *Salmonella* serotype Typhimurium strains, 25 *Salmonella* serotype Typhimurium monophasic variant strains, 25 *Salmonella* serotype Anatum strains, 12 *Salmonella* serotype Tilburg strains, 7 *Salmonella* serotype Virchow strains, 6 *Salmonella* serotype Choleraesuis strains, and 1 *Salmonella enterica* serotype 4,5,12:−:− strain). The results were expressed as the colorimetric and turbidimetric changes (in percent) and were used to enhance the classical biotype profile by adding kinetic categories. A hierarchical cluster analysis was performed by using the enhanced profiles and resulted in 14 clusters. Six major clusters grouped 94% of all isolates with a similarity of ≥95% within any given cluster, and eight clusters contained a single isolate. The six major clusters grouped not only serotypes of the same type but also phenotypic serotype variations into individual clusters. This suggests that metabolic kinetic reaction data from the biochemical tests commonly used for classic *Salmonella enterica* subsp. *enterica* biotyping can possibly be used to determine the relatedness between isolates in an easy and timely manner.

Salmonella enterica subsp. *enterica* is responsible for the vast majority of cases of salmonellosis in mammals. Classification of isolates belonging to this subspecies is usually achieved by serotyping and phage typing (3, 18). Further determination of relatedness between strains most often requires the application of molecular biology techniques, particularly when the epidemiological relatedness among isolates is to be ascertained (1, 2, 10, 14, 16). However, this is not required for most clinical or surveillance purposes; and determination of serotypes, phage types, and antimicrobial resistance patterns is still of major importance (4, 8, 15). Even though these classification methods are used worldwide, they are tedious and not routinely performed by all laboratories.

Biochemical profiling is a fast and accurate method for the identification of bacteria when it is performed with an automated system, but it is commonly disregarded as a means of grouping *Salmonella* isolates because most serotypes within a given subgroup display a very uniform biochemical reaction profile. For instance, for *Salmonella enterica* subsp. *enterica*, only serotypes Typhi, Paratyphi A, Choleraesuis, Gallinarum, and Pullorum have a distinct biochemical behavior (9). It has, however, been demonstrated that serotype Typhimurium variants that have been categorized by means of phage typing can be further differentiated by means of certain biotyping methods (6, 18).

Until now, biochemical profiling has relied on a set of biochemical tests for which a given serotype or isolate can yield either a positive or a negative result after a given incubation time. This approach, although proven and very valuable, does not take into account the rate or the kinetics with which the biochemical reaction takes place and thus neglects a circumstance that can be of biological relevance. For example, from an ecological perspective, the amount of time that an isolate requires to transform or to use a metabolic substrate may influence whether or not it can establish itself in a new niche, namely, in the gut of an animal. The time that bacteria require to complete a growth cycle is a variable that depends on many factors, both nutritional and genetic (11). If nutritional factors do not vary and environmental conditions are constant, only genetic factors should be of relevance when the behavior of microbial growth is studied. We assume that bacteria should then demonstrate a specific metabolic kinetic profile, taking into consideration characteristics such as their ability to adapt to the environment by making only those gene products that are essential for their survival, as well as their ability to develop sophisticated mechanisms to regulate metabolic pathways.

We examined the kinetics of 28 biochemical tests commonly used to identify members of the family *Enterobacteriaceae* for 135 *Salmonella* isolates using an automated biotyping system (Vitek). This system, in conjunction with the GNI+ card, provides stable environmental conditions and culture media and yields periodic readings of metabolic changes. The objective of

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TABLE 1. Results of metabolic tests obtained by using Vitek GNI+ system after examination of 135 *Salmonella enterica* isolates of pig origin

Test[a]	% of strains examined in the following metabolic category[b]:				
	Very fast (1)	Fast (2)	Slow (3)	Very slow (4)	Negative (0)
2,4,4′-Trichloro-2′-hydroxy-diphenylether resistance	0	0	0	0	100
Polymyxin B resistance	0	0	0	0	100
Oxidative metabolism of:					
Glucose	69	23	6	1	1
Lactose	0	0	0	0	100
Maltose	91	3	4.5	0	1.5
Mannitol	30.5	54	11	4.5	0
Xylose	29	50	4.5	0	16.5
Fermentative metabolism of:					
Raffinose	0	0	0	0	100
Sorbitol	23	6	64.5	5.5	1
Sucrose	0	0	0	0	100
Inositol	0	0	0	50[c]	50
Adonitol	0	0	0	0	100
Glucose in presence of *p*-coumaric acid	22	65	12	1	0
Rhamnose	7.5	75.5	11	1.5	4.5
L-Arabinose	28	47	17	2	6
Glucose	27.5	71	1.5	0	0
Use of:					
Acetamide	0	0	0	0	100
Citrate	44.5	50	1.5	0	4
Malonate	0	0	0	0	100
Esculin hydrolysis	0	0	0	0	100
Indoxyl-β-D-glucoside metabolism	0	0	0	0	100
Urease	0	0	0	0	100
Tryptophan deaminase	0	0	0	0	100
β-Galactosidase	0	0	0	0	100
Arginine dihydrolase	11	51	30	8	0
Lysine decarboxylase	12.5	10.5	1.5	73.5	2
Ornithine decarboxylase	30.5	60	9.5	0	0
Hydrogen sulfide (H_2S) production	43	30.5	21.5	0	5

[a] The GNI+ card contains 30 biochemical tests, whereas 2 tests are used for control purposes (growth and decarboxylase enzyme).
[b] Categories established according to isolates kinetic reaction to 28 biochemical tests. The numbers in parentheses represent the category numbers described in Materials and Methods.
[c] As inositol results are not reproducible and the positive or negative reaction for all tests cannot be determined until 10 h, all positive tests are categorized as very slow.

this study was to determine if metabolic kinetic data can be used to biotype isolates with a higher discriminatory power than the classical biotyping method, allowing rapid determination of strain relatedness.

MATERIALS AND METHODS

***Salmonella* strains.** One hundred thirty-five *Salmonella* strains isolated from pig samples (from 1997 to 2002) were randomly chosen from a bacterial collection kept at the Veterinary Faculty of the Universitat Autònoma de Barcelona. All except one of the isolates originated from Spain; a single *Salmonella* serovar Choleraesuis isolate came from Germany. The resulting serotype distribution was as follows: serotype Typhimurium ($n = 59$), monophasic serotype Typhimurium strains (4,5,12:i:− ($n = 25$), serotype Anatum ($n = 25$), serotype Tilburg ($n = 12$), serotype Virchow ($n = 7$), serotype Choleraesuis ($n = 6$), and serotype 4,5,12:−:− ($n = 1$). The 59 serotype Typhimurium isolates included phage types DT 104b ($n = 11$), DT 104 ($n = 6$), DT U302 ($n = 6$), DT 208 ($n = 4$), DT 193 ($n = 2$), DT 41 ($n = 2$), DT 110 ($n = 1$), and nontypeable ($n = 27$). The 25 monophasic variant serotype 4,5,12:i:− isolates included phage types DT U302 ($n = 17$), DT 208 ($n = 1$), DT 193 ($n = 1$), DT 120 ($n = 1$), as well as nontypeable isolates ($n = 5$). This distribution is roughly representative of the serotypes isolated in our laboratory from 1997 to 2002. All isolates were epidemiologically unrelated and originated from different farms, and some had been used in previous studies (5, 12).

Culture and biochemical data. Selected isolates were seeded onto blood agar and incubated for 24 h at 37°C. A 1.0 McFarland suspension was prepared by turbidimetric adjustment in 0.45% sterile saline solution for each isolate. Gram-negative organism identification cards (GNI+; bioMérieux Vitek, Marcy l'Étoile, France) were then inoculated and incubated in a Vitek Jr. system (VJS; bioMérieux). These cards contain 28 biochemical tests (Table 1) plus two additional

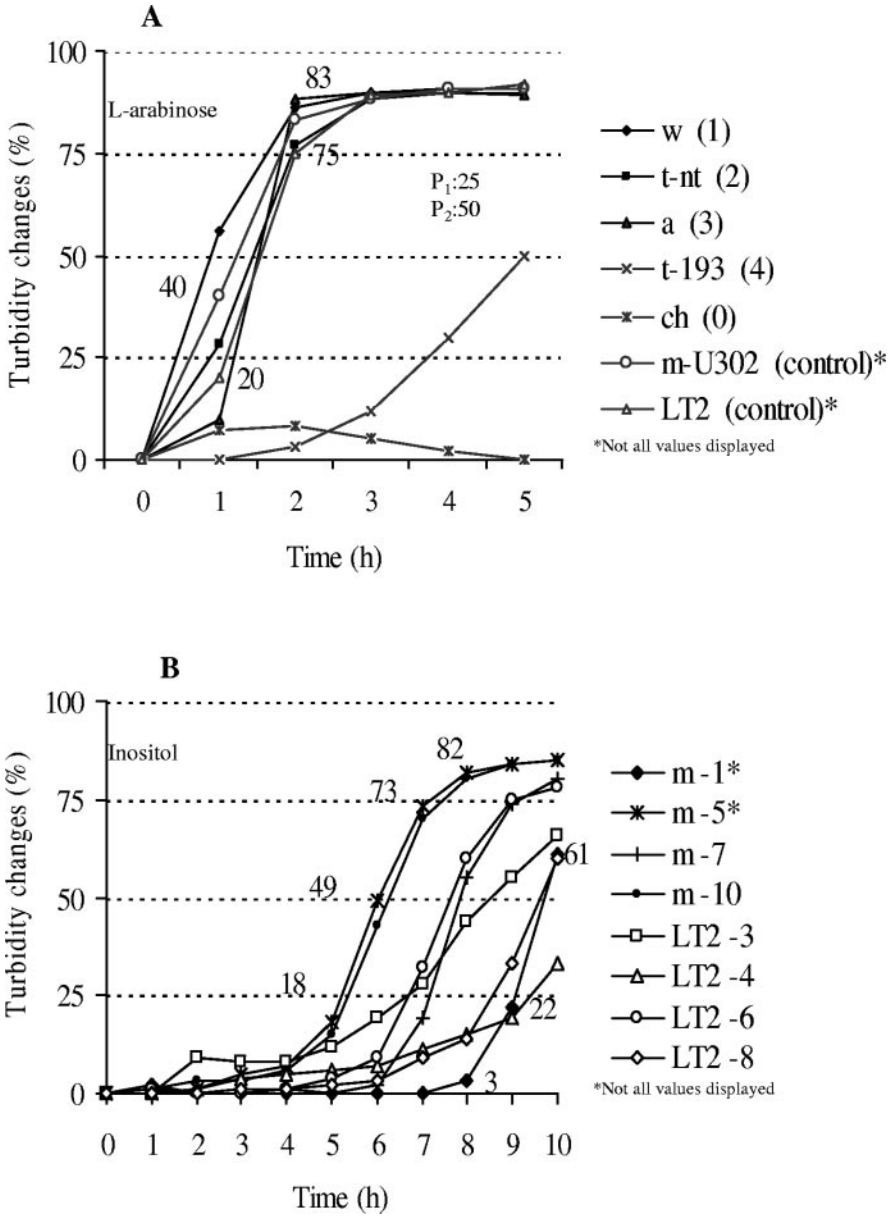

FIG. 1. Results of two biochemical tests displaying the turbidimetric and colorimetric percent changes for *Salmonella enterica* isolates. (A) L-Arabinose fermentation test results for five isolates exemplifying the five categories (shown as abbreviated serotype and phage type) and two control strains; (B) inositol fermentation test results for four replicas of each control strain.

tests for control purposes (growth and decarboxylase enzyme). VJS performed readings of each test by means of a photometric sensor that evaluated the turbidimetric or colorimetric changes and analyzed the data by using bioLiaison software (BioMérieux). The results were expressed as a percentage of transmittance reduction and were compared to the reading at time zero. This process was repeated every 60 min. The final readings were made at 18 h.

Validation of biochemical kinetics reproducibility. Two *Salmonella* strains were used for control purposes: *Salmonella* serotype Typhimurium LT2 (serotype reference strain) and a randomly chosen monophasic serotype Typhimurium variant 4,5,12:i:− isolate. The control strains were analyzed by VJS on two consecutive days (five replicas per day, with each replica originating from a distinct colony). Regression curves (time versus light transmittance change) were calculated for each test and strain. In order to evaluate the reproducibility of the method, the regression curves were statistically compared by curvilinear estimation by using a logarithmic model.

Profiling of biochemical test rates. Since the results obtained with VJS were found to be reproducible based on the criteria established for this study, all isolates were tested only once. According to the recommendations of the manufacturer, a strain was considered positive for a given test if the percentage of turbidimetric or colorimetric change (at 12 h of incubation) was ≥25% of that measured from time zero. Tests that were negative for all isolates ($n = 13$) were discarded from further analysis. Raw kinetic data were used to create a correlation matrix by using the similarity distance method via Pearson's coefficient (SPSS Inc., Chicago, IL). By considering a correlation coefficient of 0.80 as a cutoff, SH_2 production, rhamnose fermentation, and citrate utilization were found to be correlated ($r > 0.80$; $P < 0.05$), as were mannitol fermentation and ornithine decarboxylation ($P < 0.05$). Subsequently, only citrate utilization and mannitol fermentation were considered for further analysis. All other tests were considered independent of each other.

Strains were classified according to the time required to reach certain color-

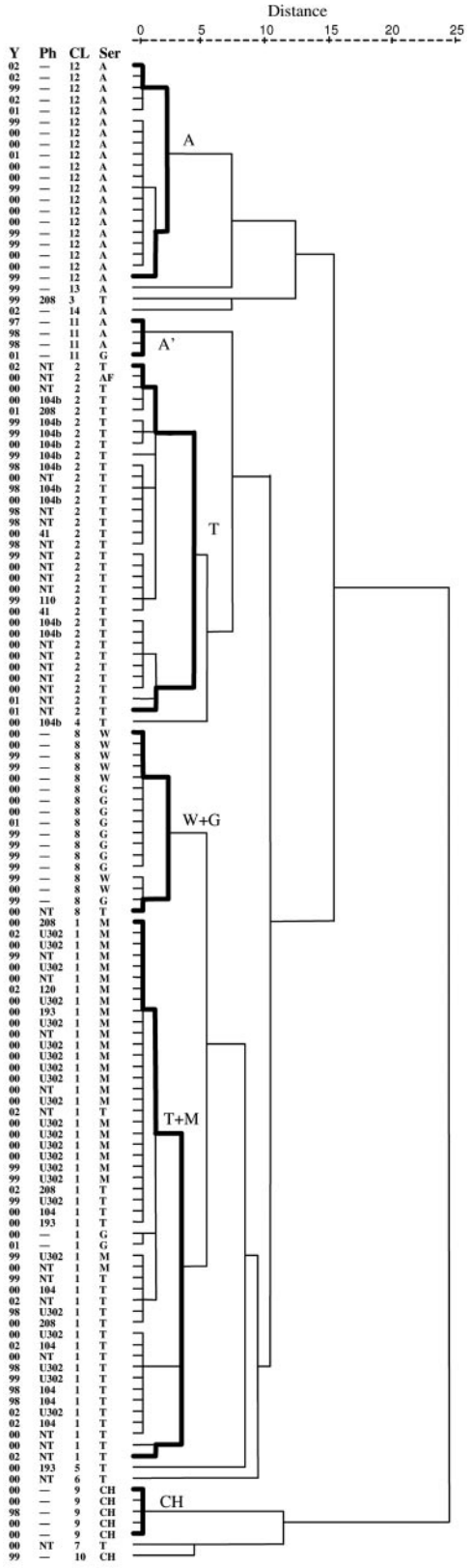

FIG. 2. Dendrogram depicting a hierarchical cluster analysis of 135 isolates originating from *Salmonella enterica* serotypes after being categorized by means of metabolic kinetics. Y, year of isolation; Ph, phage type (displayed only for serovar Typhimurium and the serovar Typhimurium monophasic variant); Cl, cluster; Ser, serotype (AF serotype 4,5,12:−:−); A, serovar Anatum; A′, old serovar Anatum; T, serovar Typhimurium; W+G, serovars Virchow and Tilburg; T+M, serovar Typhimurium and serovar Typhimurium monophasic variant; CH, serovar Choleraesuis.

imetric and turbidimetric change rate values. These values, which corresponded to two specific curve points, were chosen according to the results obtained from the two reference strains. The first point corresponded to a colorimetric or turbidimetric change rate range ≥25% (the positive cutoff for a given test) and <50%; the second point corresponded to a change rate ≥50% but within the exponential curve phase.

Isolates were categorized in a comparative ranking by using these curve points. Category 1 (Table 1) was assigned to those isolates that reached the ≥50% change first. Category 2 was assigned to the isolates that reached the ≥50% change in second place, after having reached ≥25% change at an earlier time. Category 3 grouped those isolates that reached ≥50% change in third place or those that reached ≥50% change in second place but that did not reach ≥25% change at an earlier time. Category 4 was assigned to isolates that reached ≥25% change but that never reached ≥50% change or that reached 50% at a very late point in time. Category 0 was assigned to isolates that did not reach a 25% change rate (negative). All possible cases were taken into consideration by using this categorization model (Fig. 1A). For practical purposes, category 1 was named "very fast," category 2 was named "fast," category 3 was named "slow," and category 4 was named "very slow."

Statistical analysis and biotyping. The relationship between isolates was established by using a hierarchical cluster analysis. Clusters were determined by using the average linkage between groups and were calculated by using the squared Euclidian distance method (SPSS v. 12.0). The SPSS application grouped the isolates into 14 clusters based on isolate similarities of ≥95%. Parallel cluster analyses were performed for control purposes by using points randomly chosen from within the 25% to 40% and 50% to 75% change rate range.

RESULTS

Reproducibility. When the raw percentage data were used, all test results (10 replicas × 15 positive tests × 2 sample strains = 300 curves) were reproducible except for inositol fermentation (Fig. 1B). Consequently, inositol fermentation results were categorized as only positive or negative. The intraday reproducibility was very high ($R^2 = 0.97$), while the reproducibility for assays run on different days was somewhat lower ($R^2 = 0.71$). However, when the categories were used, the reproducibility was 1 in both cases.

Biochemical kinetics and categorization time line. The fastest positive reactions were observed for glucose oxidation and fermentation and for mannitol oxidation. For these tests strains could be assigned to a category within 3 h of incubation. Seven other tests (lysine decarboxylation; citrate utilization; L-arabinose, sorbitol, glucose [*p*-coumaric acid] fermentations; and xylose and maltose oxidation) allowed strains to be categorized after 5 to 6 h. Seven hours of incubation was required to categorize the strains for arginine dihydrolase, and 10 h of incubation was required to determine inositol fermentation results.

Clustering and biotyping. Fourteen clusters were created. Six major clusters contained 94% of all isolates ($n = 127$). The serotype distribution within these clusters was as follows: cluster A included 20 serotype Anatum isolates from 1999 or later; cluster A′ included 3 serotype Anatum isolates from 1997 and 1998; cluster T included 31 serotype Typhimurium isolates; cluster W+G comprised 1 serotype Typhimurium isolate, 7 serotype Virchow isolates, and 8 serotype Tilburg isolates; cluster T+M included all monophasic serotype Typhimurium

variants plus 22 serotype Typhimurium isolates; and cluster CH included 5 of the 6 serotype Choleraesuis isolates, all of which originated in Spain. The other eight clusters each contained a single isolate (Fig. 2).

As far as the most encountered *Salmonella* serotype Typhimurium phage types (104, 104b, and U302) are concerned, their distribution within the six major clusters was as follows: 10 of 11 *Salmonella* serotype Typhimurium 104b isolates were in cluster T, and the other 104b isolate formed a single cluster. Phage types 104 and U302 were found only in cluster T+M (n = 29). Nontypeable serotype Typhimurium isolates were found in both clusters T and T+M (18 and 12 isolates, respectively).

The biotype profile for cluster T+M was characterized by very fast or fast kinetics and by being inositol positive. Cluster W+G isolates were also inositol positive. The other four major clusters were inositol negative. Cluster CH displayed a slow or very slow kinetic biotype for most tests. Interestingly, lysine decarboxylase activity was found to be very slow for clusters with a fast profile and very fast for cluster CH isolates.

The similarity of isolates within the same cluster was at least 95%, with the similarity reaching 99% in clusters CH and A′. The similarity within cluster T+M was ≥96%. This value was higher (98%) when monophasic serotype Typhimurium isolates were considered separately. The similarity between clusters was variable, whereas isolates of the CH cluster were the least similar to isolates of the other major clusters (75%).

The use of alternative curve points, as described in Materials and Methods, produced very similar clustering results, with less than 10% variance in isolate categorization.

DISCUSSION

The identification and reporting of *Salmonella* occurrences is important for surveillance purposes and for the study of outbreaks. Identification is usually accomplished by serotyping and phage typing, which is not routinely done by all laboratories. Finding a method that can tentatively place an isolate in an epidemiological context expeditiously and with an acceptable degree of accuracy would be very useful. The goal of this study was to evaluate a biotyping method that uses biochemical kinetic data obtained from an automated system which yields results in 12 h to 18 h. This method is not to be seen as a replacement of existing typing methods but, instead, as an additional means of determining isolate relatedness in a timely manner.

Classical biotyping considers two categories for each test, positive or negative, and can only differentiate *Salmonella* subspecies or very distinct serotypes (serotypes Typhi, Paratyphi A, Choleraesuis, Gallinarum, and Pullorum). Classification of isolates according to their rate of biochemical activity instead of the consideration of only positive or negative results may enhance the discriminatory power of biotyping and might reveal characteristics of ecological or epidemiological importance.

The conduct of a comparative kinetic study of 135 isolates—consisting of 28 biochemical tests per isolate—required the reproducibility of the results as well as a method that could be used to compare the resulting curves for a specific test type (22). In our study the first requirement was fulfilled by using JVS, an automated system that has proven to be accurate (13, 19, 23), guarantees stable test conditions (Vitek system and GNI+ cards), and provides reproducible test results (R^2 = 0.97), according to the criteria defined for this study, as described in Materials and Methods.

In order to find the best possible typing method, we first had to determine an algorithm that matched the resulting curves (>2,000 curves). After examination of the curves, it became clear that a different algorithm would be required for each test, and sometimes even within the same test, resulting in an enormous amount of data that would be impossible to manage. As a consequence, it was decided that only the exponential phases of the curves were of relevance and that these could be approximated by using two control points within this phase: the cutoff point and a second point that represents a higher degree of change. Even though the two points for a certain test type were arbitrarily chosen, a parallel cluster analysis displayed that the resulting correlation between isolates was practically identical, as long as the points were within this predefined range.

All strains were previously categorized by using serotyping and phage typing methods. Comparison of those results to the results obtained by use of the enhanced biochemical profiles confirmed that this method has a high discriminatory power. For example, *Salmonella* serotype Typhimurium phage types 104 and U302 and phage type 104b were allocated into two distinct groups, respectively. These three phage types are the most frequently encountered in *Salmonella enterica* serotype Typhimurium isolates, whereas phage type U302 is the most commonly found in the serotype Typhimurium monophasic variant 4,5,12:i:− (7, 20, 21). Previous studies have already reported that phage types 104 and U302 are closely related (17), while phage types 104 and 104b are less related (5). This method, however, was not able to discriminate between isolates belonging to *Salmonella* serotypes Virchow and Tilburg. Closer examination showed that their biochemical kinetic profiles differed only in a single test category (arginine dihydrolase) and that this difference was not significant enough to separate the isolates.

Enhancement of the kinetic profile by the addition of additional biochemical tests might increase the discriminatory power of our method, allowing it to distinguish between isolates of distinct serotypes. It cannot be discounted that this might also disperse the results, making their interpretation less clear, even though the correlation between our results and the results obtained by serotyping and phage typing suggest otherwise; this will have to be evaluated by further studies.

In conclusion, we believe that our results and the potential of this method merit further studies and believe that this line of study should include an increased number of strains and biochemical tests. Should these studies validate our method, it can possibly be used to rapidly establish relationships between *Salmonella* isolates in an outbreak scenario.

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